Fumarate hydratase-deficient renal cell carcinoma is strongly correlated with fumarate hydratase mutation and hereditary

Fumarate Hydratase-deficient Renal Cell Carcinoma is Strongly Correlated with *Fumarate Hydratase* Mutation and Hereditary Leiomyomatosis and Renal Cell Carcinoma Syndrome

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Abstract

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) syndrome-associated RCC are difficult to diagnose prospectively. We used immunohistochemistry (IHC) to identify FH (Fumarate Hydratase)-deficient tumors (defined as FH negative, 2-succinocysteine (2SC) positive) in cases diagnosed as "unclassified RCC, high grade or with papillary pattern", or "papillary RCC type 2", from multiple institutions. 124 tumors (from 118 patients) were evaluated by IHC for FH and 2SC. An FH deficiency was found in 24/124 (19%) cases. An indeterminate result (only one marker abnormal) was found in 27/124 (22%) cases. In a TMA of 776 RCCs of different types, only 2 (0.5%) tumors, initially considered papillary type 2, were FH-deficient. *FH* mutations were found in 19/21 FH-deficient tumors (9 confirmed germline) and in 1/26 FH indeterminate tumors identified by IHC. No *FH* mutations were found in 2/21 FH-deficient RCC, 25/26 FH indeterminate RCC and 10/10 RCC demonstrating FH expression by IHC. Patients with FH-deficient RCC had median age of 44 years (range 21 to 65). Average tumor size was 8.2cm (range 0.9 to 18cm). FH-deficient RCC were characterized by at least focal macronucleoli and demonstrated two or more growth patterns in 93% cases. Papillary was the most common (74%) and dominant (59%) pattern, while other common patterns included: solid (44%), tubulocystic (41%), cribriform (41%) and cystic (33%). At presentation, 57% were stage ≥pT3, 52% had positive nodes, and 19% had distant metastases. After mean follow-up of 27 months (range 1-114 months), 39% of patients were dead of disease and 26% had disease progression. We conclude that FH and 2SC are useful IHC ancillary tools which allow recognition of FH-deficient RCC.

Introduction

Hereditary leiomyomatosis and renal cancer syndrome (HLRCC) is an autosomal dominant disorder characterised by inherited predisposition to uterine and cutaneous leiomyomas and renal cell carcinoma (RCC). It is characterized by inactivating germ-line mutation in the *fumarate hydratase* (*FH*) gene, which is located at 1q42.3-q43 and codes for an enzyme involved in the tricarboxylic acid cycle, which hydrates fumarate to form malate. (1-6) Although the hereditary association with multiple leiomyomas of the skin has been known for more than 60 years now,(7) the first syndromic association of uterine and skin leiomyomas with renal carcinoma was reported in 2001 in two families from Finland.(2, 5) HLRCC-associated RCC was included in the 2004 WHO classification of renal neoplasms (8), however not as a distinct RCC subtype, but as a presumed hereditary counterpart of papillary RCC type. Subsequent publications highlighted the aggressive behaviour of the renal carcinomas associated with HLRCC syndrome and expanded its morphologic spectrum, emphasizing the presence of orangiophilic or eosinophilic macronucleoli with perinucleolar halos (viral inclusions-like).(9, 10) HLRCC syndrome-associated RCC is currently recognized as a separate entity in the 2013 International Society of Urological Pathology (ISUP) Vancouver Classification of renal tumors(11) and it is included in the upcoming WHO classification 2016.

Biallelic inactivation due to *FH* mutations in HLRCC syndrome results in either complete loss or reduction of the FH enzymatic activity, which leads to accumulation of the intracellular levels of fumarate.(4, 12) The increased level of fumarate modifies the cysteine residues in many proteins, resulting in increased protein succination and production of S-(2-succino)-cystein (2SC), which can be used as a marker for detection of these FH-deficient tumors.(13) Accumulation of high

levels of 2SC, resulting in positive 2SC immunohistochemistry (IHC) staining, was shown to be highly specific for detection of HLRCC-associated RCC.(14) The loss of FH enzymatic activity, resulting in negative fumarate hydratase staining on IHC was also demonstrated to have a high specificity in identifying HLRCC-associated tumors.(15, 16) However, a combined IHC approach to investigate the utility of both 2SC and FH antibodies in detecting previously unknown HLRCC-associated RCC has so far not been reported.

Although the primary morphologic pattern described in HLRCC-associated RCC is papillary, these tumors have been shown to demonstrate many growth patterns, often in combination, which can also present a diagnostic challenge when evaluating neoplasms with unknown clinical or familial background.(9, 10, 14) Because of their rarity and diagnostic difficulties in identifying these tumors, we postulated that many of them are currently under recognized, under reported or misclassified and we sought to evaluate the utility of IHC for 2SC and FH in identifying these tumors, particularly in cases signed out either as "unclassified RCC, high grade", "unclassified RCC with papillary pattern", or "type 2 papillary RCC". We also describe the pathological features, *FH* mutational status and the clinical features of RCCs demonstrating lack of FH expression (*fumarate hydratase-deficient RCC),* characterized by FH negative and 2SC positive staining on IHC.

Material and Methods

An institutional Ethics Review was obtained for the study.

Pathology identification of cases

We initiated an international collaboration and deliberately searched for renal neoplasms labelled in the initial sign-out as "unclassified RCC, high grade" or "unclassified RCC with papillary pattern" or type 2 papillary RCC. In particular, we searched for tumors exhibiting: 1) aggressive features, such as invasion into perirenal or sinus fat and/or showing regional metastatic disease; 2) at least focal macronucleoli, and 3) presence of different growth patterns, all of which were previously associated with HLRCC-associated RCC. Although many of the participating collaborators had large in-house and consult practices with subspecialty interest in urologic pathology, the search of the respective institutional databases was subject to varying digital archive limitations for retrospective searches. All potential cases were reviewed by two urologic pathologists and a representative tissue block was retrieved for additional studies. We also included 2 previously confirmed and published HLRCC-associated RCC.(17, 18) Clinicopathologic and follow-up data were collected on cases demonstrating IHC profile compatible with FH deficiency, by review of the institutional records and by contacting the consulting pathologists.

TMA evaluation of papillary RCC enriched cohort

Using tissue microarray (TMA) methodology, a total of 776 renal neoplasms from three separate institutions were evaluated by IHC for FH and 2SC. TMAs were enriched for papillary RCC (381), comprising 175 papillary RCC type 1, 68 type 2, 39 mixed and 99 papillary RCC, not specified. TMAs also included other renal tumor types: clear cell RCC (232), chromophobe RCC (21), oncocytoma (25), other RCC (39), and urothelial carcinoma (78). TMAs were constructed using 0.6 or 1 mm cores in duplicate or triplicate for each neoplasm (with built-in controls).

FH and 2SC Immunohistochemistry

IHC for FH and 2SC was performed in one laboratory on formalin fixed paraffin embedded (FFPE) sections. We used a commercially available primary anti-FH mouse monoclonal antibody (1 in 2000 dilution; clone J-13, cat no sc-100743, Santa Cruz Biotechnology, USA) and an anti-2SC rabbit polyclonal antibody (1:2000, antibody provided by Dr. Norma Frizzell) on an automated staining platform—the Leica Bond III Autostainer (Leica Biosystems, Mount Waverley, Victoria, Australia). For FH, heat induced epitope retrieval (HIER) was performed for 30 minutes at 97 degrees Celsius in the manufacturer's alkaline retrieval solution ER2 (VBS part no: AR9640). For 2SC, HIER was performed for 30 minutes at 97 degrees Celsius in the manufacturer's acidic retrieval solution ER1 (VBS part no.AR9961).

FH and 2SC IHC were scored independently by two pathologists (KT and AG) on whole slide sections from tumors with "unclassified, high grade", or "unclassified with papillary pattern" diagnosis, as per study design. Absent staining for FH in the neoplastic cells, in the presence of a positive internal control in blood vessels, inflammatory cells, other stromal cells, and non-neoplastic cells of the kidney parenchyma was interpreted as true negative staining (loss or FH-deficient status). All other patterns of staining were considered positive, provided the staining was cytoplasmic and granular (that is mitochondrial). Staining for 2SC on cases evaluated on whole section was scored as negative (0); (1+) if focal (<50% of cells reactive) or diffuse (>50% of cells reactive) but of weaker intensity; or (2+) if diffuse positive (>50% of cells reactive) with moderate to strong intensity. In positive 2SC cases, we also attempted to localize the reactivity (cytoplasmic, nuclear or both). Negative staining in the adjacent normal renal parenchyma was considered an internal negative control. Due to the limited amount of available tissue in the TMA evaluated cases, 2SC was only scored as negative or positive.

Positive IHC result was considered when both antibodies showed pattern indicating FH deficiency (FH -, 2SC 2+); negative IHC result was when FH antibody showed retained FH expression and 2SC was not expressed (FH +, 2SC 0). Indeterminate IHC result, was considered when only one of the markers showed aberrant expression status suggesting FH deficiency, while the other was equivocal or negative (FH +, 2SC 1+ to 2+; or FH -/+, 2SC 2+).

Molecular evaluation of FH mutations

All cases with available tissue that demonstrated FH deficiency (FH -, 2SC 2+) or showed indeterminate result (only one antibody reactive), underwent molecular evaluation of *FH* gene mutation status by Sanger sequencing and loss of heterozygosity (LOH) studies on DNA extracted from macrodissected FFPE tissue. We also evaluated 10 cases with retained FH expression for *FH* mutation, as a negative control group. For Sanger sequencing previously described custom primer sets were used and the whole coding sequence including exon-intron junctions was sequenced using primers designed to produce short amplicons suitable for degraded formalin-fixed DNA.(19) LOH studies were performed using a previously described set of six polymorphic short tandem repeat markers (D1S517, D1S2785, D1S180, AFM214xe11, D1S547 and D1S2842), surrounding the *FH* gene.(19) Additional patients with suspected HLRCC were offered *FH* germline testing as part of their clinical care. This clinical genetic testing was performed using massively parallel sequencing for small nucleotide variants with Sanger confirmation and Multiplex Ligation-dependent Probe Amplification (MLPA) for detection of large scale deletions.

Results

Pathology evaluation of cases by FH and 2SC immunohistochemistry

Over 3000 cases from multiple institutions were reviewed to select possible study cases that fulfilled the search criteria (spanning the years 1996-2014). We identified 124 tumors (from 118 patients) for additional IHC evaluation by FH and 2SC using representative whole slide sections. Most of the re-reviewed cases not included in the study remained "unclassified" after review, but additional IHC or other testing was not performed on excluded cases to further assess their classification. An IHC result indicating FH deficiency (FH -, 2SC 2+) was found in 24/124 (19%) cases (Fig. 1A-C) and 73/124 (59%) showed retained FH function by IHC (FH +, 2SC 0) (Fig. 1D-F). Indeterminate IHC profile, when only one of the markers was suggestive of FH deficiency (FH + with 2SC 1+ to 2+; or FH -/+ with 2SC 2+) was found in 27/124 (22%) cases (Fig. 2A-F). All FH negative cases (FH score 0) also demonstrated diffuse strong positive staining for 2SC (2SC 2+). In the FH-deficient cases, 2SC (2+) was diffusely and strongly positive in the cytoplasm in all cases; in only 35% of cases a distinct nuclear staining could be confirmed, while in the remaining cases the nuclear staining was difficult to evaluate due to strong cytoplasmic reactivity. In the cases considered indeterminate for FH expression, 2SC reactivity was restricted only to the cytoplasm.

TMA evaluation by FH and 2SC immunohistochemistry

Of the TMA evaluated cases, only 2/381 (0.5%) papillary RCC, both initially considered type 2 (pt #2 and #17) (3% of all type 2 papillary RCC), showed FH deficient result (FH -, 2SC 2+) (Fig. 3A-C). All other tumor types evaluated on TMA, which included 232 clear cell RCC, 21 chromophobe RCC, 25 oncocytomas, 39 other RCC, and 78 urothelial carcinomas demonstrated

retained FH expression, which was somewhat variable, but typically of moderate to strong intensity, while the corresponding 2SC staining was considered negative in all evaluated cases (FH+, 2SC 0).

Molecular evaluation of FH mutations

The IHC results for FH and 2SC for FH-deficient cases and the corresponding *FH* mutational alterations are shown in Table 1. We performed molecular testing on 64 cases, of which 57 produced informative result. We analyzed 21/26 cases considered FH-deficient by IHC (24 identified on whole slide and 2 on TMA by IHC). We also evaluated 26/27 cases considered FH indeterminate by IHC and 10/73 cases which showed retained FH expression by IHC.

Mutations were found in 19/21 FH-deficient tumors, while in 2 cases mutations could not be identified (one had low DNA quality and in neither case were large scale deletions sought by MLPA). 9/19 cases underwent germline testing and the *FH* mutations were confirmed germline in all tested cases, while the remaining 10/19 cases, which harboured *FH* inactivating mutations, had only neoplastic FFPE tissue available for testing and germline mutations could not be confirmed. Of 26 FH indeterminate tumors, only 1 case demonstrated *FH* mutation with IHC profile: FH -/+, 2SC 2+ (Figure 2D-F), but no specific germline testing was performed. The remaining 25/26 demonstrated wild type *FH* (IHC profile: 21 (FH +, 2SC 1+); 3 (FH +, 2SC 2+); and 1 (FH -, 2SC 2+). All 10 cases with normal FH function (FH+, 2SC 0) by IHC showed wild type *FH*.

Clinicopathologic findings in FH deficient RCC

The clinico-pathologic findings in 27 FH-deficient RCC are shown in Table 2. They were almost twice as common in men (M:F=1.9:1), with a median patient age of 44 years (mean 44; range 21 to 65 years). Twenty-one patients were Caucasians; one patient each was Asian and African-American. Skin leiomyomas were documented in 3/23 (13%) patients and 5/8 (63%) female patients had prior uterine leiomyomas; of note, FH was also negative by IHC in 2/2 females with tested uterine leiomyomas. Family history of either renal tumors, skin or uterine leiomyomas was elicited in 6/23 (26%) patients. Overall, an association with HLRCC syndrome was documented in 8/23 (35%) patients, based on the presence of skin and uterine leiomyomas in the patients or their kindreds, familial history of syndromic features, and *FH* mutational alterations. There was a predilection for the left kidney (L:R=1.5:1). Solitary tumors were found in 21/23 (91%) patients and 2 patients had bilateral tumors (one had multiple neoplasms in both kidneys). Average tumor size was 8.2cm (median 8.5cm, range 0.9 to 18cm). 57% of patients had stage ≥pT3 and 52% had positive nodes at surgery; in 19% patients distant metastatic disease (M1) was also found at presentation. After a mean follow-up of 27 months (median 17.5, range 1-114 months), 39% (9/23) patients were dead of disease and 26% (6/23) had disease progression, with evidence of local recurrence or subsequent regional or distant metastases.

The 27 FH-deficient neoplasms were characterized by variable and different architectural growth patterns and typically two or more patterns were found in 93% of cases, as shown in Table 3. Although papillary pattern was most commonly present and seen in 74% of cases (in 59% as a dominant one), other common patterns were also seen: solid in 44%, (dominant in 22%), tubulocystic in 41% (dominant in 7%), cribriform in 41% (dominant in 4%) and cystic in 33% (not seen as dominant). Tubulopapillary was a dominant pattern in 1 case (4%); 1 (4%) case

showed sarcomatoid differentiation as a dominant morphology. Examples of different morphologic growth patterns are illustrated in Figure 4A-F. By design, all cases demonstrated at least focal macronucleoli, which in some cases were ubiquitous. In cases with papillary pattern, typically there was absence of foam cells in the fibrovascular cores; hyalinization of the fibrovascular cores was noted in 9 (45%) cases.

Discussion

In this study we demonstrated that one fifth of RCC, diagnosed either as "unclassified RCC, high grade" or "unclassified RCC with papillary pattern", are FH-deficient by IHC and were almost invariably accompanied by *FH* mutations. Only 0.5% of all cases diagnosed previously as papillary RCC, and 3% of those considered type 2 papillary RCC, showed FH-deficiency by IHC and *FH* mutations, while all other evaluated renal tumors showed retained FH expression. The FH-deficient RCCs shared remarkable clinico-pathologic similarities with HLRCC-associated RCC, including younger age at presentation, aggressive clinical behaviour and adverse morphologic features, and were characterized predominantly by papillary architecture, typically admixed with other growth patterns, with invariable presence of at least focal macronucleoli. In fact, we were able to document an association with HLRCC syndrome in 8/23 (35%) of patients. By IHC, these tumors typically demonstrated FH-deficient profile (FH -) with aberrant succination, resulting in diffuse and strong 2SC reactivity (2+), which has previously been shown to be strongly associated with HLRCC-related RCC.(13, 14) The IHC profile, along with the morphology, aggressive clinical behaviour, and the presence of *FH* mutations, provide strong justification to consider these tumors as part of the spectrum of the HLRCC-associated RCC.

We believe that both antibodies, FH and 2SC, should be used simultaneously to enhance the IHC potential in detecting FH-deficient RCC. Combined negative staining for FH and strong positive staining for 2SC demonstrated very good sensitivity for FH-deficient RCC profile and excellent specificity. That is, a normal pattern of staining for FH and 2SC can be used to rule out FH deficiency in the great majority of renal carcinomas encountered. However, we identified 2 tumors (patient #19 and #21, 2nd tumor) with variably retained FH expression by IHC; both tumors showed *FH* point mutations (2SC 2+ in both cases). As previously shown, possible missense or other in-frame *FH* mutations may be associated with retained FH expression, resulting from a synthesis of a stable, but inactive enzyme.(13-15, 20) In addition, all cases considered "indeterminate" on IHC due to 2SC 1+ (21 cases) or 2SC 2+ (3 cases), but showing retained FH expression (FH +), exhibited wild type *FH*. Therefore, restricting the evaluation to only one of the antibodies would be limited by the relatively lower sensitivity of FH and the lower specificity of 2SC. We also found it difficult to reliably confirm if 2SC reactivity was nuclear, when diffuse and strong cytoplasmic reactivity was present. This is in contrast to Chen Y-B et al(14) who found both cytoplasmic and nuclear 2SC to be present in HLRCC-associated RCC, allowing them to distinguish it from the "cytoplasmic only" pattern observed in a proportion of papillary RCC, type 2 and some unclassified, high grade RCC cases. The clinical utility of the 2SC antibody is also currently limited, because it is not yet commercially available and cannot be routinely used in surgical pathology laboratories. Given the lower specificity and the difficulty in interpretation of 2SC, negative FH appears to be a more specific and comparably sensitive test at the present time.

Recent studies have also shown that IHC reactivity for 2SC(21) or 2SC in combination with FH (20, 22) aid in identifying FH-deficient leiomyomas in younger patients, associated with HLRCC syndrome. We have previously shown that although the great majority of patients with HLRCC syndrome will have FH deficient leiomyomas, 1% of all sporadic uterine leiomyomas are FH deficient usually due to somatic inactivation.(22) This is in contrast to the current study, where germline *FH* mutations were identified in all patients with FH-deficient RCC with sufficient material for testing.

The RCC associated with HLRCC syndrome have been reported in about 30% of HLRCC families.(4, 12) HLRCC-associated RCC are particularly difficult to manage because they are highly aggressive and present with advanced stage and metastatic disease, resulting in death of disease in 40-50% patients.(9, 10) Therefore, active surveillance is not recommended for the management of even small HLRCC-associated renal tumors in families with HLRCC syndrome, and wide surgical excision is recommended when any renal tumor is detected.(10) RCC associated with HLRCC syndrome are however quite rare and clinically challenging to diagnose in practice, because patients frequently do not exhibit the whole spectrum of the clinical presentations and the family association is either unknown or not apparent; clinical manifestations can also differ within families (9, 10) The initial report described renal tumors in 32% of the patients, all with metastatic disease at presentation,(2) with a prevalence of 14% reported by the National Cancer Institute (NCI) group in a North American cohort.(4) Kidney cancers have lower penetrance than the skin or uterine leiomyomas in the HLRCC affected families and they typically occur more than a decade later.(4, 9, 12) Therefore, patients may initially present only with skin or uterine leiomyomas or less commonly with renal cell

carcinoma, and renal tumors may demonstrate a delayed presentation or the patients may lack the other HLRCC syndromic features. In the largest cohort of 38 patients with renal tumors, reported by Merino et al from the NCI, 39% had documented skin leiomyomas and 55% had uterine leiomyomas.(9) The morphology remains crucial in recognizing these tumors in routine practice.

In a recent study of comprehensive molecular characterization of papillary RCC, 3.1% (5/161) of all papillary RCC and 8.3% (5/60) of those diagnosed as papillary type 2, demonstrated germ-line or somatic *FH* mutations, which were associated with the CpG Methylator Phenotype (CIMP).(23) Similar to our study, these patients were younger at presentation, and had a lower probability of overall survival than other patients with papillary RCC. A subset of these papillary RCC type 2 tumors, designated as CIMP-associated, shared the FH-deficient profile observed in HLRCC-associated RCC, based on their molecular features, allowing for more accurate characterization, which may lead to disease-specific targeted therapies.(23) This also highlights the fact that the differential diagnosis of FH-deficient RCC will typically include papillary RCC type 2, which is a relatively common renal tumor. However, the frequent papillary morphology in combination with additional architectural patterns, and at least focal presence of macronucleoli, should be regarded as morphologic clues to undertake additional IHC testing for FH and 2SC in this setting.

Currently, there is a lack of uniformly accepted definition of HLRCC-associated RCC, which is defined not just by mutational analysis, but also clinically. The NIH definition requires that the diagnosis of HLRCC is established with the identification of a heterozygous pathogenic variant in *FH* in combination with multiple cutaneous leiomyomas, with at least one histologically

confirmed leiomyoma, a single leiomyoma in the presence of a positive family history of HLRCC, and/or one or more tubulo-papillary, collecting-duct, or papillary type 2 renal tumors with or without a family history of HLRCC. For the time being, "*FH-deficient RCC"* may be the most appropriate nomenclature for tumors that show IHC negative staining for FH and strong 2SC reactivity, in the setting of uncertain clinical and family history and unknown genetic status.(24) Taking a pragmatic approach, we would recommend that if FH-deficient RCC is diagnosed, the possibility of HLRCC should be first considered clinically. If there is a suggestive personal or family history, a presumptive diagnosis of HLRCC can be made pending confirmation with formal genetic counselling and germline mutation testing. When FH-deficient RCC is diagnosed in the absence of features suggesting syndromic disease, there is little data to indicate the risk of germline mutation. Based on our limited data (the finding of germline *FH* mutation in all 9 patients who had sufficient material available for testing), at this stage we believe the risk of germline mutation (that is HLRCC) is very high, and therefore performing genetic counselling and mutational analysis would be appropriate in all patients with FH-deficient renal carcinoma. This recommendation may be modified in the future if, similar to FH-deficient uterine leiomyoma, a low rate of germline mutation is found in follow-up studies.

The tumorigenic effect of mutated *FH* results in fumarate accumulation, which acts as a metabolic tumor suppressor, resulting in a metabolic shift toward aerobic glycolysis with decreased oxidative phosphorylation (so-called Warburg effect). It has been postulated that this alteration has possible downstream effects by inhibiting the hypoxia inducible factor prolyl hydroxylase and increasing the hypoxia-inducible factor 1 alpha (HIF1alpha), which targets vascular endothelial growth factor (VEGF), erythropoietin (EPO) and glucose transporter

1(GLUT1), and produces additional epigenetic alterations of genome-wide histone and DNA methylation, leading to increased cell proliferation and tumorigenesis.(3, 24-26) On a molecular level, these changes have also been characterized by increased oxidative stress and activation of the NRF2-antioxidant response elements (ARE) pathway.(23, 27, 28)

Limitations of this study include its retrospective nature, which allowed us to confirm association with HLRCC syndrome only in about a third of patients with FH-deficient RCC. For example, in some cases we were not able to obtain a dermatological confirmation of skin leiomyomatosis, and a complete family history on specific HLRCC features was not available. Although mutational analysis was performed on FFPE neoplastic tissue in the majority of tested cases, a formal *FH* genetic testing was done only in a subset of cases, perhaps with a selection bias towards patients with a high likelihood of familial disease, limiting the ability to confirm germline *FH* mutations. In 2 FH-deficient cases by IHC, we could not confirm the presence of *FH* mutations; one of the 2 cases demonstrated low DNA quality and additional studies to investigate for possible *FH* mutations in these 2 cases, for example, by MLPA to screen for large scale *FH* deletions were not performed.

In summary, we found that a substantial number of cases considered either as “unclassified RCC, high grade” or “unclassified RCC with papillary pattern”, and small percent of cases diagnosed as papillary RCC type 2, demonstrated FH-deficient pattern (FH -, 2SC 2+) by IHC and were invariably accompanied by *FH* mutations at the molecular level. Although we could document an unequivocal association with HLRCC syndrome in only about a third of the patients, there is clearly a high likelihood of syndromic disease in patients presenting with FH-deficient RCC.

Furthermore, even apparently sporadic FH-deficient RCC show striking clinico-pathological similarities to unequivocally HLRCC syndrome-associated renal carcinomas, including a younger age and adverse features at presentation, aggressive clinical behaviour, and frequent papillary architecture in combination with other growths patterns, with invariable presence of at least focal macronucleoli. In addition to the careful morphologic evaluation, IHC for FH and 2SC is a useful aid that allows recognition of the RCC with FH-deficient profile with FH IHC being more specific and 2SC being more sensitive.

Figure legends:

Figure 1

An FH-deficient RCC showing a papillary growth pattern (A). The cells exhibit eosinophilic macronucleoli with perinucleolar clearing (inset) (patient #6). On IHC, neoplastic cells demonstrate FH negative staining, while non-neoplastic cells show granular cytoplasmic staining, used as positive internal control (B). 2SC shows diffuse and strong staining in the neoplastic cells (2+) (C). In cases with retained FH expression, such as in this example with papillary growth pattern (D), FH shows diffuse staining in the neoplastic cells (E), while 2SC is negative (F).

Figure 2

Some cases demonstrated indeterminate IHC profile for FH, with only one of the markers suggestive of FH deficiency while the other was equivocal or negative. In this example showing papillary growth (A), FH demonstrated retained expression (B), while 2SC showed variable staining pattern (1+) (C); no *FH* mutation was identified on molecular analysis. In another example with indeterminate IHC profile (D), FH showed focal expression (E), but 2SC was diffusely positive (2+) (F). On molecular analysis, *FH* mutation was identified in this case (patient #19).

Figure 3

We identified 2 cases on TMA with FH-deficient pattern, originally considered papillary RCC type 2 (A). Both cases showed prominent eosinophilic nucleoli (inset). In both cases, FH was negative (B), while 2SC was strongly positive (C). *FH* mutation was confirmed in the illustrated tumor from the patient #2.

Figure 4

FH-deficient RCC were characterized by various architectural growth patterns with two or more patterns present in great majority of cases. Papillary pattern was most commonly present as a dominant one (A) and hyalinization of the papillary fibrovascular cores was frequent (B). In this example, an admixed tubulocystic pattern is also seen (right) (B). Other frequent patterns included solid, in this example with foci resembling collecting-duct carcinoma (C), cribriform (D), cystic, which in this example also shows intracystic papillary growth (E) and tubular, which in this example shows multiple intracytoplasmic vacuoles imparting admixed cribriform morphology (F).

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Tables

TABLE 1. FH and 2SC immunohistochemistry and *FH* molecular alterations in fumarate hydratase-deficient RCC

Patient	FH IHC	2SC IHC	*FH* mutation status
1	-	+ +	c.1189G>A, p.Gly397Arg
2	-	+ +	c.174_177dupTGAAA, p.Leu60Ter
3	-	+ +	c.496G>T, p.Gly166Ter
4	-	+ +	Large scale deletion of whole of *FH* on MLPA (Germline)
5	-	+ +	c.413_414del, p.Leu138fs (Germline)
6	-	+ +	Large scale deletion of *FH* and OPN3 gene on MLPA (Germline)
7	-	+ +	c.689A>G, p.Lys230Arg (Germline)
8	-	+ +	NA (pt deceased before consent)
9	-	+ +	c.239dupA, p.Ile81AspfsTer14
10	-	+ +	Negative
11	-	+ +	c.911_917 del CTTTTGT(Phe 305Leufs*22)
12	-	+ +	NA
13	-	+ +	c.1385_1390+6del
14	-	+ +	NA
	-	+ +	Negative (low quality DNA)
15	-	+ +	LOH positive (*FH* wt, sample with abundant non-tumor tissue)
16	-	+ +	c.395_398delTAAAT, p.Leu132Ter
17	-	+ +	c.805delA, p.Ile269fsTer15
18	-	+ +	NA
19	-/+	+ +	c.139C>T, p.Gln47Ter
20	-	+ +	c.320A>C, p.Asn107Thr (Germline)
21	-	+ +	c.698G>A, p.Arg233His (Germline)
	+	+ +	c.698G>A, p.Arg233His
22	-	+ +	c.1189G>A, p.Gly397Arg (Germline)
	-	+ +	c.1189G>A, p.Gly397Arg (Germline)
	-	+ +	c.1189G>A, p.Gly397Arg (Germline)
23	-	+ +	NA (pending, recent case)

TABLE 2. Clinical characteristics of patients with fumarate hydratase-deficient RCC

Pt	Sex	Age	Leiomyomas	Family History	Side	Greatest size (cm)	pTNM stage	Metastasis	Follow-up (mo)	Status
1	M	65	No	Unk	L	18	T3aN1	Liver, lung, spleen and bone at presentation	3	DOD
2	M	62	Unk	Unk	L	10	T2aN0		114	AND
3	M	60	No	Unk	R	8	T2aNX		7	AND
4	F	25	Uterine at 25 (FH-)	Yes*	R	4	T1aNX		17	AND
5	M	44	Unk	Yes†	R	4.5	T3aN0		7	AND
6	M	25	Skin	Yes‡	R	14	T3aN1	Liver, flank wall at 6 months	64	DOD
7	F	32	Uterine	Yes§	L	3	T1aNX	Left para-aortic lymph nodes	56	AWD
8	M	35	Unk	Unk	R	10	T3aN1	Para-aortic lymph node	18	DOD
9	M	51	Unk	Unk	R	14	NA		96	AND
10	M	46	Unk	Unk	L	10	T3aN1	Bone (multiple)	24	DOD
11	M	44	Unk	Unk	NA	8	NA	Lung, lymph nodes at 6 months	6	AWD
12	F	40	Unk	Unk	L	9	T3aN1	Peritoneum, retroperitoneum, lymph nodes, omentum	24	DOD
13	M	52	Unk	Unk	R	14	T4N1	Lung, mediastinum	13	DOD
14	M	41	No	Unk	L**	1	T1aNX	Similar hilar tumor resected after 1 year (see below)	13	AWD
		42	No	Unk	L	4	T3aN1	Perihilar lymph node	13	
15	F	21	No	Yes¶	L	5.5	T3aNX		12	AND
16	M	42	Unk	No	L	10	T2N0	Aorta involvement	18	DOD
17	M	21	No	Unk	L	5	T4N1M1	Bone (rib), retroperitoneal nodes	4	AWD
18	M	46	Unk	Unk	NA	NA	T3bN1M1	NA	12	DOD
19	F	50	Unk	Unk	L	10.9	T2N0		18	AND
20	F	59	Skin, uterine	Unk	L	12.5	T4N1M1	Liver, lung, supraclavicular and iliac lymph nodes (direct into adrenal, pancreas)	1	DOD
21	F	51	Skin, uterine at 31	Yes#	L	1.4††	T1aNX		46	AWD
		51			R	0.9	T1aNX		46	
22	M	56	No	Unk	R	3.5‡‡	T3aN0		31	AWD
	M	56	No	Unk	L	9	T2aNX			
	M	57	No	Unk	R	4	T1aNX	Probable local recurrence (same side tumor)		
23	F	43	Uterine at 38 (FH -)	Unk	L	12.5	T3aN1M1	Bone (tibia)	1	AWD

Abbreviations: DOD=died of disease, AWD=alive with disease, AND=alive no disease, NA=not available, Unk=unknown

*Uncle had kidney removed in 2009 for multiple tumours; †Twin brother died from metastatic 'kidney cancer'; ‡Mother with multiple uterine leiomyomas, grandfather died of renal carcinoma and had multiple skin lesions (not further characterised); §Mother and one sister tested positively for *FH* gene; ¶Father and paternal grandmother with "skin lesions"; #Maternal aunt with "renal tumors"; **Two similar RCC resected from left kidney (second after 12 mo); ††Bilateral multiple renal tumors; ‡‡Bilateral renal tumors, second tumor on in the right kidney possibly local recurrence

TABLE 3. Morphologic patterns seen in fumarate hydratase-deficient RCC. Dominant patterns are marked by asterisk.

Case	Papillary	Solid	Tubulocystic	Cribriform	Cystic	Tubular	Sarcomatoid	Tubulopapillary	# of Patterns
1		+*	+				+		3
2	+*	+			+	+		+	5
3	+*		+	+	+				4
4		+	+*		+				3
5	+	+*				+		+	4
6	+*								1
7	+*	+			+				3
8	+*				+			+	3
9	+*				+				2
10	+*		+						2
11				+*		+			2
12	+*			+	+	+			4
13		+*				+	+		3
14	+	+*	+						3
15	+*	+	+		+				4
16	+*					+			2
17	+*		+	+					3
18	+*	+							2
19								+*	1
20	+*					+			2
21		+					+*		2
22	+*		+	+					3
23	+*		+	+					3
24	+	+*		+					3
25	+*		+		+				3
26		+*		+			+		3
27	+		+*	+					3
Total	20/27 (74%)	12/27 (44%)	11/27 (41%)	11/27 (41%)	9/27 (33%)	7/27 (26%)	4/27 (15%)	4/27 (15%)	

Figure 1

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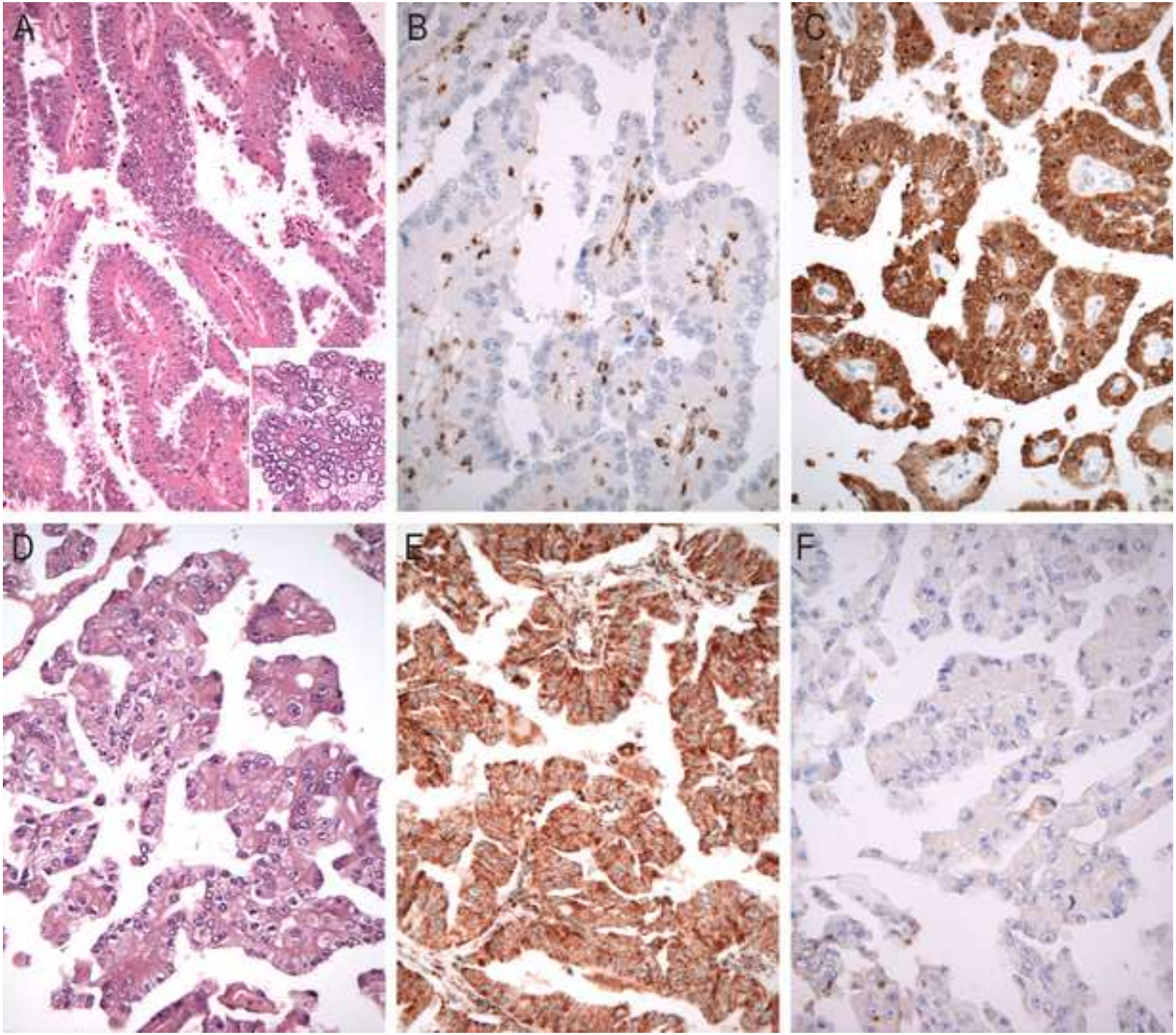

Figure 2

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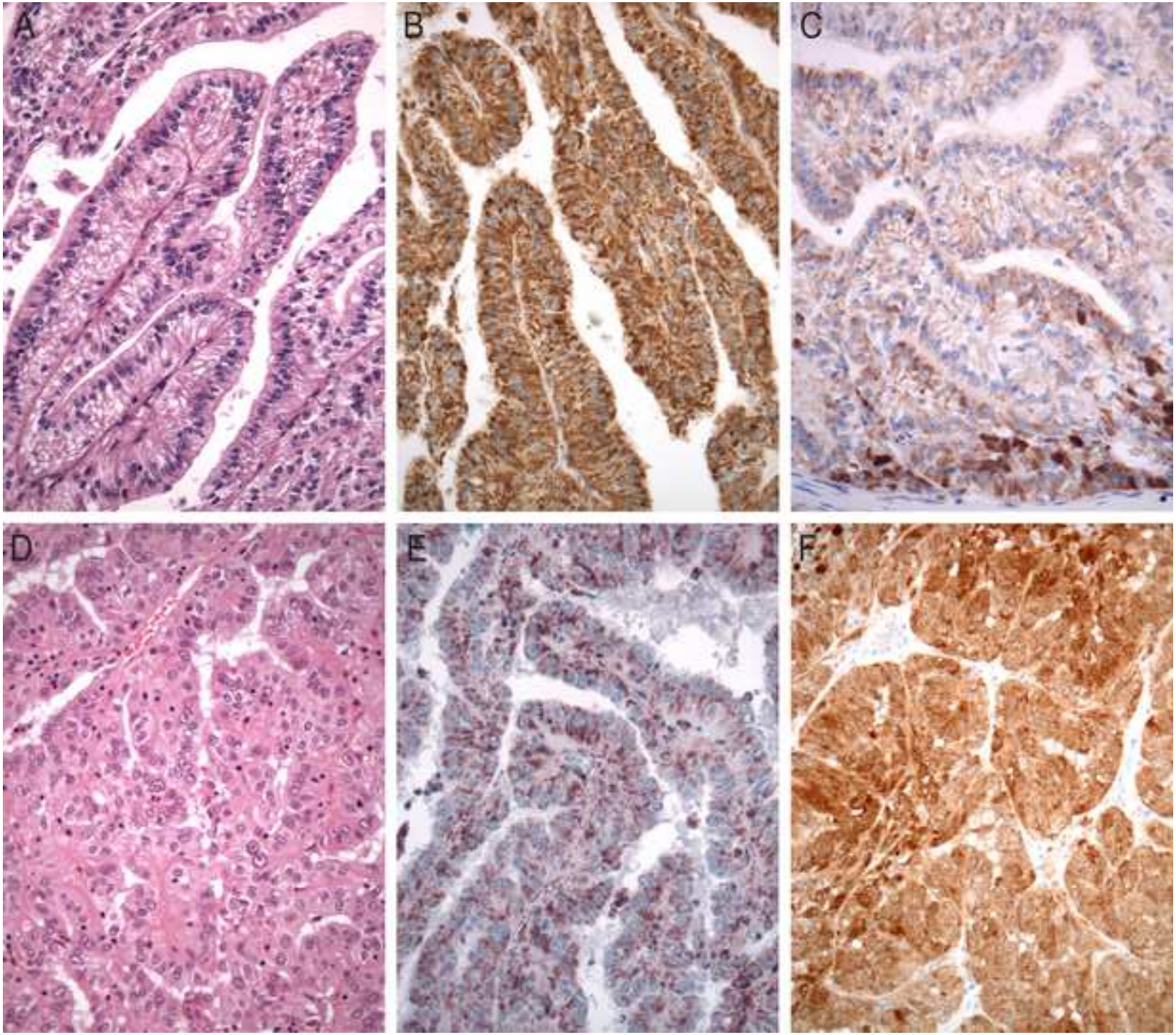

Figure 3

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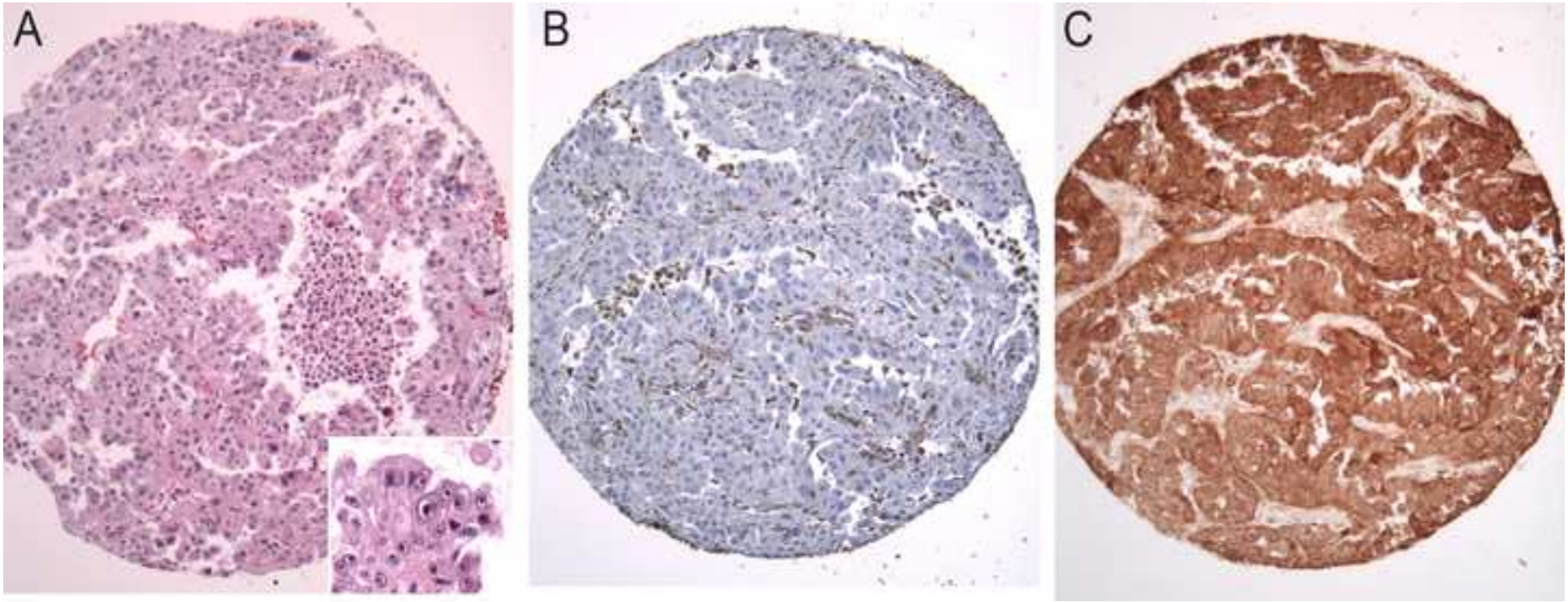

Figure 4

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